IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Yu *et al.*
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Title : **BROAD SPECTRUM ANTI-VIRAL THERAPEUTICS AND PROPHYLAXIS**

Art Unit : 1652
Examiner : Saidha, Tekchand
Customer No.: 20985

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Sir:

I, Fang Fang, declare as follows:

1) I am the President of Research and Development at Nexbio, Inc., located at 10665 Sorrento Valley Road, San Diego, California 92121. I earned an M.D. degree from Peking University Medical College, and a Ph.D. degree in Cell Biology from the University of California, San Diego.

2) In my position at Nexbio, Inc., I have extensively been involved in or directed projects assessing the ability of sialidases and compounds containing a sialidase linked to a glycosaminoglycan (GAG)-binding domain to protect cells from being infected by viruses.

3) Using materials and methods described in the above-referenced application and standard methods as described herein, myself and other scientists under my direction performed experiments to test the effectiveness of compounds containing a human sialidase domain and a GAG-binding domain against a variety of strains of influenza virus. As exemplified by the results shown and discussed below, we have demonstrated that, by following the teachings in the application, one can (i) prepare compounds containing a human sialidase domain and a GAG-binding domain, where the resulting compounds retainin sialidase enzymatic activity; and (ii) use a compound containing a human sialidase domain and a GAG-binding domain to protect cells from being infected by a variety of strains of influenza virus.

MATERIALS AND METHODS

A. *Constructing and testing a human sialidase – GAG-binding fusion protein.*

A compound containing a human sialidase domain and a GAG-binding domain was made using standard molecular cloning methods:

Compound 1, AR-G_{4S}-Neu2 (G_{4s} linker is 4 glycines, followed by a serine), was constructed using the GAG-binding sequence of human amphiregulin (AR) linked at the C-terminus to a sequence encoding a five-amino-acid linker (GGGGS), which in turn was linked at the C-terminus to the gene encoding the human sialidase NEU2 (Monti, E, Preti, Rossi, E., Ballabio, A and Borsani G. (1999) *Genomics* 57:137-143). The construct was cloned into the pTrc99a expression vector.

B. *Production of Compound 1*

To produce Compound 1, the expression construct was transformed into *E.Coli* BL21. A single colony was inoculated into 2.5 ml of LB broth and grown overnight at 37°C with shaking. In the morning, 2 ml of overnight culture was inoculated into 500 ml of TB medium in a 2 liter shake flask and the culture was allowed to grow to OD_{600}=4.0 (2-4 hours) at 37°C with shaking. Protein expression was induced by addition of IPTG to a final concentration of 1 mM and continued for 3 hr with shaking. Cells were harvested by centrifugation at 5,000xg for 10 min. Cells were washed once (resuspended in PBS and recentrifuged) and resuspended in 15 ml of lysis buffer.

The bacterial cells suspended in lysis buffer were lysed by sonication, and the cell debris was removed by centrifugation. Clarified lysate was passed through an SP-Sepharose column (bed volume 15 ml, flow rate 120 cm/hour). The column was reconditioned to lower pH and salt with one volume of PBS to ensure good retention of the protein during endotoxin removal. Endotoxin was removed by washing the column with 5 volumes of PBS containing 1% Triton X-100, 0.5% Sodium Deoxycholate and 0.1% SDS. The detergents were washed away with 3 volumes of PBS and 3 volumes of lysis buffer. Proteins were eluted from the column with lysis buffer that contained 0.8 M NaCl. The fraction eluted from SP-Sepharose was adjusted to 1.9 M $(NH_4)_2SO_4$ (most contaminating proteins are salted out at this step) and clarified by centrifugation. The supernatant was loaded onto a Butyl-Sepharose column (flow rate 120

cm/hour). The column was washed with 2 volumes of 1.3 M $(NH_4)_2SO_4$ and the fusion was eluted with 0.65 M $(NH_4)_2SO_4$. For the final step, size exclusion chromatography was performed on Sephacryl S-200 equilibrated with PBS buffer at a flow rate of 25 cm/hour. Sialidase activity was determined using the standard enzyme-linked lectin assay (ELLA). Protein concentration was determined using Bio-Rad's Bradford kit. Protein purity was assessed by SDS-PAGE and estimated to be >98%.

C. *Cell Protection Assay testing Compound 1*

Stocks of Influenza Viruses

Influenza viral strains were obtained from ATCC and the repository at St. Jude Children's Research Hospital. All experiments involving influenza viruses were conducted at Bio-safety level II.

Viruses were propagated on Madin-Darby canine kidney (MDCK) cells in minimal essential medium (MEM) supplemented with 0.3% bovine serum albumin and 0.5 micrograms of trypsin per ml. After incubating for 48 to 72 hours, the culture medium was clarified by low speed centrifugation. Viral particles were pelleted by ultracentrifugation through a 25% sucrose cushion. Purified viruses were suspended in 50% glycerol-0.1M Tris buffer (pH 7.3) and stored at -20^0C.

Cell protection assay

To evaluate the ability of Compound 1 to protect cells against influenza viruses, MDCK cells were treated with Compound 1 and the cells were then challenged with a broad selection of human influenza viruses (IFV) including human IFV A of H1, H2 and H3 subtypes, human IFV B as well as an avian IFV strain. To perform the assay, MDCK cells were treated with milliunits (mU) of Compound 1 as recited in **Table 1**, at 37 °C for 2 hrs. The cells were subsequently challenged with the various strains of influenza viruses at MOI 0.1 for 1 hr. The cells were washed and incubated in fresh DMDM:F12 supplemented with 0.2% ITS (GIBCO) and 0.6 μg/ml acetylated trypsin (Sigma). The cells were stained with 0.5% crystal violet and 20% methanol for 5 min and rinsed with tap water. The level of viable cells in each well was quantitated by extracting crystal violet by 70% ethanol and reading at 570 nM. Cell protection was calculated using the formula:

$$100 \times \frac{\{(V\text{sialidase treatment followed by viral challenge} - V\text{viral challenge alone})\}}{\{(V\text{sialidase treatment alone} - V\text{viral challenge alone})\}}$$

where **V** is the number of viable cells after each of the above treatments

RESULTS

The ability of Compound 1 to remove sialic acid from cell surfaces was confirmed by lectin ELLA assay.

Table 1 shows the results of cell protection assays conducted using Compound 1; Letters in bold describe the influenza strain used in the assay.

Table 1 : Results of cell protection assays conducted using Compound 1:

Influenza Strain	**Compound 1 /well (mU)**	**Cell Protection by Compound 1**
A/WS/33	90	49%
A/PR/8/34	90	29%
A/turkey/Wis/66	90	12%
A/Victoria/504/2004 (H3N2)	2	46%
A/Japan/305/57 (H2N2)	2	51%
A/HongKong/8/68	2	18%
B/Lee/40	90	12%

The results show that a compound containing a human sialidase domain and GAG-binding domain protects the cells against a variety of strains of influenza virus.

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I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application of any patent resulting there from.

Fang Fang

Date: Dec 22, 2009